TregFlowEx Kit

Cat.No. ED7417

Description

The TregFlowEx Kit is designed for the detection of regulatory T-cells (CD4+CD25+FOXP3+ cells) in human peripheral blood or human umbilical cord blood using flow cytometry.
Regulatory T-cells (Treg cells) are a subset of lymphocytes that have immune suppressive properties and play a critical role in the maintenance of self-tolerance. Their regulatory function leads to protection of tissues from collateral damage triggered by immune responses against microbes and allergens, they facilitate maternal tolerance to allogeneic fetus during pregnancy and maintain homeostasis with commensal microbiota[1].
Tregs are usually characterized as CD4+ CD25high T-cells expressing forkhead box transcription factor (FOXP3). Such phenotype is found in 5-12% of human peripheral blood CD4+ T-cells[2]. Tregs can be further distinguished into the so-called "natural" Tregs originating in thymus (tTreg cells) and the peripherally induced Tregs (pTreg cells)[3]. The discrimination between tTregs and pTregs is still disputable, however several proteins had been proposed to be the tTreg defining marker: the transcription factor Helios and Neutropilin-1, the receptor for proteins of the vascular endothelial growth factor family[4].

Specification

Fix and Lysing Solution (10x concentrated) contains formaldehyde based fixative that causes fixation of leukocytes together with erythrocyte lysis.
Permeabilizing Solution (ready-to use) contains a buffered mixture of deteregents that permeabilizes cellular membranes.
Blocking Buffer (ready to use) contains a mixture of proteins that block non-specific binding of antibodies during intracellular staining.
CD4 FITC/CD25 PE (ready to use) contains a cell surface staining reagent comprising of a stabilized cocktail of mouse monoclonal antibody against human CD4, FITC labelled (clone MEM-241, isotype IgG1) and mouse monoclonal antibody against human CD25, R-PE labelled (clone MEM-181, isotype IgG1).
FOXP3 APC (ready to use) contains an intracellular staining reagent comprising of mouse monoclonal antibody against FOXP3, APC labelled (clone 3G3, isotype IgG1).

Reagents provided

ED7417-1 **Fix and Lysing Solution**, 1 x 10 ml
ED7417-2 **Permeabilizing Solution**, 1x 25 ml
ED7417-3 **Blocking Buffer**, 1 x 2.5 ml
ED7417-4 **CD4 FITC/CD25 PE**, 1 x 0.5 ml
ED7417-5 **FOXP3 APC**, 1x 0.25 ml

Materials required but not provided

Deionized water (dH_2O)
Phosphate buffered saline (PBS)
1% formaldehyde solution in PBS
5ml test tubes (12 × 75 mm)

Storage and handling

Store the TregFlowEx Kit at 2-8°C. Expiration date is printed on reagent labels and on the kit outer packaging label.

Warnings and precautions

- Intended for research use only.
- Do not use reagents after expiration date.
- Avoid contamination of the reagents.
- Avoid prolonged exposure of the reagents to direct sunlight.
- **Fix and Lysing Solution** (ED7417-1 contains diethylene glycol; methanol and formaldehyde.
  H phrases
  H302+312+332 Harmful if swallowed, in contact with skin or if inhaled.
  H315 Causes skin irritation.
  H317 May cause an allergic skin reaction
  H319 Causes serious eye irritation.
  H335 May cause respiratory irritation.
  H351 Suspected of causing cancer
  H371 May cause damage to organs.
  H373 May cause damage to organs (kidney) through prolonged or repeated exposure if swallowed.
  P phrases
  P270 Do not eat, drink or smoke when using this product.
  P280 Wear protective gloves / protective clothing / eye protection / face protection.
  P301+P312 IF SWALLOWED: Call a POISON Center or doctor/physician if you feel unwell.
  P302+P352 IF ON SKIN: Wash with plenty of soap and water.
  P305+P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
  P501 Dispose of contents/container to authorized facility for dangerous wastes.
  See product Safety Data Sheet for full information on the potential hazards and how to work safely with the product.
- Human blood samples are considered as potentially infectious and must be handled with care.
- The flow cytometer should be calibrated on a routine basis using fluorescent microbeads to ensure stable sensitivity of detectors.
- Flow cytometer may produce false results if the device has not been regularly calibrated and maintained appropriately.
- Data may be incorrectly interpreted if fluorescent signals were compensated wrongly or if gates were positioned inaccurately.
- Reagents were specially formulated to perform at low temperatures. It is important to adhere to the low temperature requirements throughout the procedure otherwise the fluorescence signals, the total number of cells and the number of the identified target cells will decrease considerably.
- In case of reagent deterioration or if data obtained show any performance alteration, please contact manufacturer using following e-mail address: technical@exbio.cz

Application

Measurement of Treg cells percent count as frequency of CD4+ lymphocytes in human whole blood using flow cytometry.

Specimen

Human anticoagulated peripheral blood and human umbilical cord blood. Store the blood samples at room temperature. The kit is not suitable for processing Ficoll purified cell suspensions (PBMCs).

Reagent preparation

Fix and Lysing Solution
Fix and Lysing Solution is provided as 10x concentrated solution that needs to be diluted with deionized water. Dilute only the necessary amount of the concentrate (1 ml of the diluted solution is needed per reaction) and store the diluted solution at 2-8°C. The diluted solution is stable for 1 month.

Permeabilization Solution
Bring Permeabilization Solution to room temperature. Check for SDS precipitation and if necessary, dissolve the SDS by warming the vial to 37°C. Afterwards store the Permeabilizing Solution at room temperature (15-25 °C). Storage at room temperature does not affect the reagent shelf life.

PBS
Prepare PBS according to recipe below (other recipes may apply).
Dissolve:
8.0 g of Sodium Chloride (NaCl)
0.2 g of Potassium Chloride (KCl)
2.0 g of Potassium Phosphate monobasic (KH2PO4)
1.42 g of Sodium Phosphate dibasic dihydrate (Na2HPO4·2H2O)
in 800 ml of deionized H2O.
Adjust the pH to 7.4 with HCl.
Add deionized H2O to 1 liter.
Sterilize by autoclaving for 20 minutes at 15 psi or by filter sterilization. Store at 2-8°C.

1% formaldehyde in PBS
Prepare 1% formaldehyde in PBS by mixing 1 part of methanol stabilized 37% formaldehyde solution from Sigma-Aldrich (Cat. No. F1635) and 36 parts of PBS. Store at 2-8°C. The solution is stable for 1 month.
Warning: The concentrated formaldehyde solution is classified as acutely toxic substance. Refer to the safety precautions provided by your formaldehyde supplier.

Required for handling

Refrigerated centrifuge
Refridgerator
Automatic pipettes with disposable tips
Vortex mixer
Flow cytometer - blue laser excitation at 488 nm, red laser excitation at 633 nm and proper emission filters
Liquid waste container with appropriate disinfectant
Vacuum connected Pasteur pipettes to aspirate supernatant during cell washes (optional)

Procedure

Surface staining
1. Add 10 µl of CD4 FITC/CD25 PE reagent to tubes.
2. Add 100 µl of human blood.
3. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8°C).
4. (optional wash) Wash cells by adding 2 ml of cold PBS and centrifuge at 300 g for 5 minutes at 4 °C.
5. Aspirate supernatant and proceed immediately to the intracellular staining.

Intracellular staining
6. Add 1 ml of the diluted cold Fix and Lysing Solution.
7. Mix and incubate for 10 minutes in the dark in the refrigerator (2–8°C).
8. Add 0.5 ml of Permeabilizing Solution.
9. Mix and incubate for 10 minutes in the dark in the refrigerator (2–8°C).
10. Centrifuge the cells at 400 g for 5 minutes at 4°C. *(Do not add any other buffer to the Fix/Perm mixture before centrifugation)*
11. Remove (decant) supernatant.
12. Add 50 µL of cold Blocking Buffer.
13. Add 50 µL of cold PBS.
14. Mix and incubate for 5 minutes in the dark in the refrigerator (2–8°C).
15. Add 5 µl of FoxP3 APC reagent.
16. Mix and incubate for 30 minutes in the dark in the refrigerator (2–8°C).
17. Wash cells twice with 2 ml of cold PBS, centrifugate at 400 g for 5 minutes at 4 °C. Aspirate/decant supernatant.
18. Add 300 µl of 1% formaldehyde in PBS and store the stained samples at 2–8°C until measured in flow cytometer. Analyze the processed samples within 4 hours.

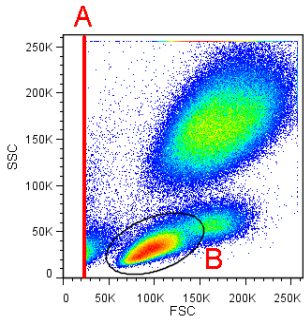
Flow Cytometric Analysis

Analyze the stained samples using flow cytometer equipped with 488 nm and 633 nm excitation lasers and the appropriate filters.
Refer to your cytometer specifications to identify the detectors in which the fluorescence of the stained cells will be collected.
Set the voltage on light scatter detectors, forward angle light scatter (FSC) and side (perpendicular) light scatter (SSC) so that the events of interest are on scale.
Set the threshold on FSC so that only cells of interest are recorded and most of the debris excluded.
Set the voltage on fluorescence detectors so that all measured events are on scale.
Analyze 10 000 – 30 000 CD4+ lymphocytes per sample. Acquire data using software intended for sample analysis supplied with cytometer.

Analysis of samples

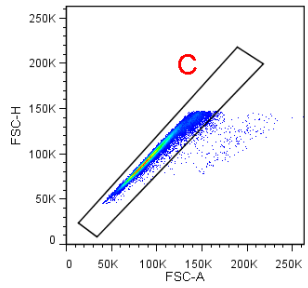
Visualize measured data as a dot-plot, where forward-scatter (FSC) is on the X-axis and side-scatter (SSC) is on the Y-axis (Figure 1). Set the target cell population gate (lymphocytes) (A) and threshold (B).

Fig. 1 Leukocyte scatter plot: threshold setting and the target cell population gate



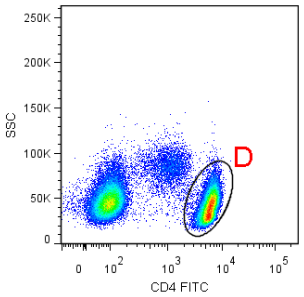
Then visualize events from the lymphocyte gate as a dot-plot, where the X-axis represents forward-scatter area (FSC-A) and the Y-axis represents forward-scatter height (FSC-H) (Figure 2). Separate the single cell population with a diagonal gate (C).

Fig. 2 Separation of single cells from aggregates



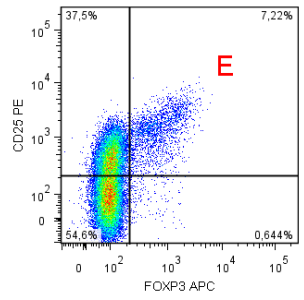
Visualize the lymphocyte single cell as a dot-plot, where X-axis represents fluorescence intensity in FITC detector and the Y-axis represents side-scatter (SSC) (Figure 3). Carefully set the gate around CD4+ lymphocytes (D).

Fig. 3 Separation of CD4+ lymphocytes (D) from CD4+ monocytes and CD4 negative cells



Visualize CD4+ lymphocytes as a dot-plot, where the X-axis represents the FOXP3 signal (fluorescence intensity in APC fluorescence detector) and the Y axis represents CD25 signal (fluorescence intensity in PE fluorescence detector). Place the gate that separates the CD4+CD25+FOXP3+events (Figure 4). Use appropriate controls to set the discrimination lines correctly.

Fig. 4 CD4+ lymphocytes displayed according to their CD25 and FOXP3 expression. The Treg cells (CD4+CD25+FOXP3+) are found in the upper right quadrant.



References

[1] Sakaguchi S, Miyara M, Constantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol (2010) 10:490–500.

[2] Churlaud G, Pitoiset F, Jebbawi F, Lorenzon R, Bellier B, Rosenzwajg M, Klatzmann D. Human and Mouse CD8(+)CD25(+)FOXP3(+) Regulatory T Cells at Steady State and during Interleukin-2 Therapy. Front Immunol. 2015 Apr 15;6:171.

[3] Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, Jiang S, Kuchroo VK, Mathis D, Roncarolo MG, Rudensky A, Sakaguchi S, Shevach EM, Vignali DA, Ziegler SF. Regulatory T cells: recommendations to simplify the nomenclature. Nat Immunol. 2013 Apr;14(4):307-8.

[4] Lin X, Chen M, Liu Y, Guo Z, He X, Brand D, Zheng SG. Advances in distinguishing natural from induced Foxp3(+) regulatory T cells. Int J Clin Exp Pathol. 2013;6(2):116-23.

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Trademarks

n/a

Revision History

• Version 1, ED7417_TDS_v1
Initial Release
• Version 2, ED7417_TDS_v2
The company logo changed. TDS layout changed. Minor changes in text phrasing. All limitations and warnings are presented together in the section Warnings and precautions. Additional symbols were implemented to product and/or components labels: "Keep away from sunlight", "For Research use only".

Symbols

REF	Catalog number
LOT	Batch code
	Use-by date
	Temperature limits
	Consult instructions for use
	Keep away from sunlight
	Manufacturer
RUO	For Research use only. Not for use in diagnostic or therapeutic procedures.

exbio

TregFlowEx Kit

50 tests | Cat.No. **ED7417**

For Research use only.
Not for use in diagnostic or therapeutic procedures.

Technical Data Sheet

Version ED7417_TDS_v2_EN
Date of Issue: 05-10-2020

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